MAURITIUS RESEARCH COUNCIL

PHYTOPLASMA DISEASES ON TOMATO IN MAURITIUS

Final Report

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MAURITIUS RESEARCH COUNCIL

Address:

Level 6, Ebène Heights,
34, Cybercity,
Ebène 72201,
Mauritius.

Telephone: (230) 465 1235
Fax: (230) 465 1239
Email: mrc@intnet.mu
Website: www.mrc.org.mu

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PI Name and Address

Dr(Mrs) Arty Gungoosingh-Bunwaree

Research Scientist/Senior Research Scientist

Plant Pathology Division

AREU

Réduit

MAURITIUS RESEARCH COUNCIL FINAL REPORT

PART I- PROJECT IDENTIFICATION INFORMATION

1. This material is based on work supported by the Mauritius Research Council under the **Unsolicited Research Grant Scheme**

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Any opinion, findings and conclusions or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the views of the MRC.

PART II: SUMMARY OF COMPLETED PROJECT (FOR PUBLIC USE)

Phytoplasmas are cell wall-less bacteria associated with more than 1000 crop diseases worldwide. The major objectives behind this study were to determine the status of phytoplasmas on tomato plantations in Mauritius, identify source(s) of phytoplasmas infections, determine whether phytoplasmas occur singly or in combination with viruses and devise disease management strategies at field level.

An islandwide survey across 79 tomato plantations revealed the widespread occurrence of phytoplasma diseases (`maladie pompon' in local jargon) in 74.6% plantations in Mauritius. Moreover although overall mean phytoplasma incidence was low (only 18.1%), the risk from such pathogens cannot be underestimated particularly due to leafhopper detected and the possibility of phytoplasma diseases spreading fast in situations of poor management practices at field.

Three phytoplasma groups namely Stolbur group SrXII, Aster yellows group SrI and Elms yellows group SrV were identified from tomato leaf and fruit samples by nested-PCR followed by RFLP techniques. Mixed phytoplasma and virus infections (PVY and TYLCV) were also detected by the ELISA technique.

Seedling production under insect-proof conditions together with good sanitation measures were recommended as management techniques for these emerging plant pathogens.

PART III: TECHNICAL INFORMATION (for program management use)

Introduction

Phytoplasmas are plant pathogenic bacteria in the class *Mollicutes*. They infect more than 1000 plant speciesworldwide and cause symptoms such as stunting, yellowing, witches` broom (proliferating shoots), phyllody (leaf-like petals and sepals), virescence (greening of floral organs), and sometimes withering of plants. These cell wall free prokaryotes are the smallest among the bacteria, both in cell size (0.1-0.8 µm in diameter) and genome size (0.5 to 1.3 Mbp). Dookun*et al*. (1999) were first to report the presence of phytoplasmas on tomato in Mauritius. This was followed by a publication from Gungoosingh-Bunwaree*et al*. (2007), who further revealed the presence of two phytoplasma subgroups namely, the aster yellows phytoplasmas belonging to the clover phyllody ribosomal subgroup 16SrI-C from tomato var*Sirius* grown in the open field in the north of the island, and ribosomal group 16SrV from tomato plants of var*Efrat* grown under hydroponics conditions at Britannia, in the south of Mauritius. Prior to these aforementioned reports, tomato plants exhibiting stunted growth or bunchy top symptoms were usually attributed to abiotic factors or phytotoxicity, when other common pathogenic organisms (viruses, bacteria, fungi) could not be detected from the symptomatic samples.

Objectives

The major objectives of the project were to:

i. Determine the incidence and occurrence of phytoplasmas on tomato on a regionwise andvarietywise basis.
ii. Determine the source of phytoplasmas identified
iii. Identify any vectors of phytoplasmas at field level
iv. Characterize the phytoplasmas involved
v. Determine whether phytoplasmas occur singly or in combination with other viruses.
vi. Eventually come up with most effective management strategies to control phytoplasma infections at field level.

Activities

i. Islandwide field survey across tomato plantations

The islandwide survey conducted from January 2010 to June 2011 across thenine districts of Mauritius covered 79 tomato plantations (67 open field and 12 protected) across 30 localities. A total of 12 tomato varieties, namely*Swaraksha*, *MST/32/1*, *Typhoon*, *Venus Rose*, *Pêche Rose*, *Epoch*, *Menara*, *Valentine*, *Franceska*,*Mathias*, *Synergie* and *V169*were surveyed. All tomato plantations visited were in flowering/bearing stage. At each visit a survey questionnaire was filled to gather maximum information on disease incidence and occurrence. Results of islandwide survey and findings of survey questionnaire are summarised in Table1.Common symptoms observed on suspected phytoplasma infected plants were: stunting, plant bushiness, purple colouration of leaves as seen in Plates 1 and 2. On some sites reduced fruit size was observed. In local planters` jargon, the bushiness and stunting symptoms shown by infected plants is called `maladiepompon'.

Phytoplasma symptoms were observed in 74.6% tomato plantations visited. Mean phytoplasma incidence recorded during the survey was 18.1%, with however peak incidences of 75% and 80% recorded under open field condition at Richelieu and Floreal respectively and peaks of 75% and 100% recorded under shadehouse conditions at RochesBrunes and Tamarin respectively. No specific trend was found between phytoplasma infection and time of the year. Highest phytoplasmaincidences were recorded at Black River (100%) whilst no phytoplasma occurrence was recorded in the district of Flacq.Moreover,tomato variety Pêche Rose was found to be less infectedbyphytoplasmadiseases compared to other locally grown tomato varieties, with phytoplasma disease incidence rarely reaching 25% at field level.A total of 256 tomato leaf isolates consisting of 177 symptomatic and 79 asymptomatic leaf samples were collected for phytoplasma studies. Ten weed samples (5 amaranthus"*brèdemalbar*" and 5 night shade"*brède martin*"samples) growing in the vicinity of symptomatic tomato plants and showing pronounced purple colouration (Plate 3) and eight green tomato fruits from different symptomatic plants were also collected. In the Plant Pathology laboratory, leaves were plucked individually for each sample and 10g lots were stored in plastic bags at -85^{o}C.

ii. Training in DNA based molecular techniques for testing of phytoplasmas

A one week hands-on training in the use of molecular techniques for the detection and identification of phytoplasmas from plant and insect samples was acquired at the Faculty of Agriculture, University of Bologna, Italy under the supervision of Prof.AssuntaBertaccini, major collaborator under the project. The major objective behind this activity was capacity building in molecular techniques.Topics covered during the training were:

i. Extraction of total nucleic acids from plant material
ii. Direct and nested PCR using different primers for detection of various phytoplasma groups
iii. Extraction of nucleic acids from reference strains (European stone fruit yellows, European aster yellows and Stolbur)
iv. Extraction of nucleic acids from insect vectors
v. Running of agarose and acrylamide gels to view PCR products
vi. Identification of phytoplasmas using published profiles

Techniques learnt enabled molecular activities under the project to be conducted with more ease and assurance at the Plant Pathology laboratory.

iii. DNA extraction, amplification of 16Sr DNA of phytoplasmas and optimisation of PCR.

Total nucleic acids were extracted from 207tomatoleaf samples (177 symptomatic and 30 asymptomatic) and 10 weed isolates. One gram of leaf midribs wereused (Prince *et al*.,1993). The protocol was also tried on eightgreen tomato fruits.Samples were pulverised in a precooled dry mortar in liquid nitrogen. Some samples were ground without any liquid nitrogen, since liquid nitrogen was not always available at the laboratory. Then8 ml of grinding buffer (K_2HPO_4.3H2O 21.7g, $K_2H_2PO_4$ 4.1g, sucrose 100g, BSA 1.5g, PVP-10 20g, 5.3g ascorbic acid, in 1L water, pH 7.6) was added. The filtrate was spun at 11 400 rpm for 30 min at 4 ^oC. The supernatant was gently poured off and 4ml extraction buffer (100 mMTris-HCl at pH 8.0, 100mM EDTA, 250mM NaCl) as well as 80 µl proteinase K (5 mg/ml in distilled water) were added to resuspendthe pellet. Moreover, 440 µl of 10% Sarkosyl was added and tubes were incubated for 2 h at 55 ^oC. The lysate was centrifuged for 10 min at 8 000 rpm at 4 ^oC to pellet

all debris. The supernatant was saved, 0.6 volume ice cold isopropanol was added to the supernatant, tubes were gently inverted and were kept at 4^{o}C overnight. The morning after tubes were centrifuged at 8 000 rpm for 15 min at 4 ^{o}C, the pellet was re-suspended in 3 ml TE buffer (10mM Tris-HCl at pH 8.0, 1mM EDTA) and 75 µl of 20% SDS along with 60 µl proteinase-K were added. After 1 h incubation at 37 ^{o}C, 525 µl of 5M NaCl and 420 µl CTAB/NaClsolution were added. Tubes were mixed thoroughly and incubated at 65 ^{o}C for 10 min. Around 2ml of chloroform/isoamyl alcohol followed by 2 ml phenol solution were added, tubes were vortexed and were centrifuged at 8 000 rpm for 10 min. The aqueous viscous supernatant was then transferred to a new tube and an equal volume of chloroform was added. Tubes were again centrifuged at 8 000 rpm for 10 min and 2.5 ml of isopropanol alcohol was added to the supernatant. Tubes were left overnight at 4 ^{o}C. The morning after, the pellet obtained after 30 min centrifugation at 4 ^{o}C, was washed in ethanol 70% and centrifuged for 10 min at 4 ^{o}C.Pellets were air driedfor 2 h and were finally suspended in 50 µl TE buffer (10mM Tris/HCl. pH7.5, 1mM EDTA).

Once DNA extraction was completed, the amount and purity of DNA harvested from each individual sample was measured on an*Eppendorf*biophotometer.DNA extracts were then diluted in TE buffer to a concentration of 100ng/µl before they were run on a 1% agarose gel in 0.5X TBE buffer. The gel was stained for 20 min in ethidium bromidesolution, destained for 15 mins and then bands were visualized under the UV transilluminator.

DNA of better quality was obtained with the use of liquid nitrogen.

Phytoplasmas were detected from total genomic DNA of tomato leaves, weed leavesand green fruits by nested PCR using published protocols (Gunderson and Lee, 1996;Schaff*et al*., 1992). However, since the original protocols could not be applied directly to reagents and primers available, optimization of concentrations of different components making up the master mix was carried out. The reaction was performed in a total volume of 25 µl and the master mix consisted of 16.3µl water, 2.5 µl 10X PCR buffer, 2.0 µl dNTPs,2.0 µl $MgCl_2$, 0.5 µl each of 20 µM forwardandreverse primers, 0.2 µl of 5U/µl *Taq*polymerase and1.0 µl template DNA. All molecular reagents were from *Fermentas*. A tube with reaction mixture devoid of DNA template was included as negative control. Phytoplasma specific universal primer pairs PI/P7 (Deng and Hiruki, 1991) were used for the first round of amplification of the 16S

rDNA.The PCR was run as follows: an initial denaturation step at 94 ^{o}C for 2 min followed by 35 cycles at 94 ^{o}C for I min, 50 ^{o}C for 2 min at, 72 ^{o}C for 3 min at and a final extension step of 72 ^{o}C for 10 min.

One µl of direct PCR product was diluted 1:30 before further amplification with nested primers R16F2n/R2 (Lee *et al*., 1993) or F1/B6 (Duduk*et al*., 2004). For further confirmation, primer pairs M1/M2 (Bertaccini*et al*., 2001) and R16(I)F1/R1were used (Lee *et al*., 1995). PCR products were electrophoresed on 1.0% agarose gel. An aliquot of 20 ng of DNA extract from reference strains of European stone fruit yellows (labelled as GSFY2 on Figures 1 and 2) and Stolbur (labelled as MOL on Figure 2) were used as positive controls. Moreover, a 1 Kb DNA marker from *Fermentas* was used as molecular ladder. The gel was stained with ethidium bromide and visualised under UV light (Figures 1&2).

No amplification was detected in the first PCR except for positive controls. Moreover, in the nested PCR, no amplification product was obtained with any of the asymptomatic tomato leaves, the weed samples nor with the negative controls. However in the nested PCR, out of 175 leaf and fruit samples tested (139 symptomatic tomato leaves, 20 asymptomatic tomato leaves, 8 tomato fruits, 8 symptomatic weed samples), 103 (58.9%) gave amplification products. These comprised 97 symptomatic tomato leaf samples (69.8%) and 6 fruit samples (75%), suggesting a good association between symptoms and presence of phytoplasmas.Thus, phytoplasmas could be diagnosed from tomato leaves and green fruits from symptomatic plants. Failure to amplify DNA from the 33.5 % symptomatic samples using PCR, may be due to inhibitors such as divalent Cu^{2+} ions originating from copper fungicides commonly sprayed on cropsand/or the low titre of the pathogen in some plants (Del Serrone*et al*., 2001). Symptoms on tomato plants might have been the consequence of mechanical damage by leaf hoppers, without any phytoplasma transmission. Galetto*et al*., (2011) reported that phytoplasma multiplication was faster under cooler conditions in insects (18-22 ^{o}C) but in plants,phytoplasma multiplication was faster under warmer conditions (22-26 ^{o}C). Therefore, failure to detect phytoplasmas from symptomatic plants could also be because sampling had been carried out under too low (<18 ^{o}C) or too high environmental temperature (>26 ^{o}C). Furthermore, survey resultssuggest a widespread distribution of phytoplasmas among local tomato plantations (detected from 8 out of 9 districts) irrespective of the variety grown.

iv. Visit of major collaborator

Prof A. Bertaccini major collaborator on the project carried out a 1-week visit to Mauritius in November 2010. Activities covered under the visit were:

i. Review of project status and finalisation of nested-PCR protocol
ii. Site visit to tomato plantations to monitor sampling technique
iii. Delivery of talks on phytoplasma diseases at the Mauritius Research Council and AREU Farmers` training school.

v. Detection of phytoplasma/virus mixed infections

Given that in some cases it can be quite difficult to symptomatically distinguish between phytoplasma and virus infections at field level, the enzyme-linked immunosorbent assay (ELISA) as described by Clark and Adams (1977), was used to detect mixed virus-phytoplasma infections. In this context, 40 symptomatic tomato leaf samples were tested the for tomato yellow leaf curl virus (TYLCV) and potato virus Y (PVY) by the Triple antibody sandwich (TAS) and Double antibody sandwich (DAS) ELISA techniques to detect TYLCV and PVY respectively. The suppliers` protocolsaccompanying the commercial antisera kits (*DSMZ*, Germany for TYLCVand *Agdia*, USA for PVY) were followed in each case. Seven out of 40 (17.5%)phytoplasma positive leaf samples were also found to be positive to PVY. Similarly, TYLCV was detected from 7.5% leaves and 5% showedphytoplasma-TYLCV-PVY mixed infections.Symptoms observed on tomato plants with mixed phytoplasma- virus infections were leaf yellowing and/or purple colouration, leaf distortions, bunchy top and stunting as seen in Plate 4.

This is the first report of mixed phytoplasma-begomovirus infection in tomato in Mauritius. In Mexico, the presence of phytoplasmas and two different begomoviruses namely tomato yellow leaf curl and tomato *chino La Paz* virus were reported in tomato and pepper byLebsky*et al*., (2011). The latter employed scanning electron microscopy (SEM) and molecular techniques to identify the causal pathogens of a yellow type disease in tomato and pepper.

vi. Analysis of PCR products, identification of phytoplasmas, and phylogeny studies

One hundredamplified PCR products obtained after amplification with primers M1/M2 and R16(I)FI/RI were forwarded to the University of Bologna for phytoplasma identification by RFLP analyses. Amplicons were digested with restriction enzymes*Tru*I,*Rsa*I *and Alu*I. The reaction mixture consisted of 8µl PCR product, 2.0 U restriction enzyme(*Fermentas*, Lithuania),1X reaction buffer and sterile distilled water. Digestion was carried out in a water bath for 3h at 37^{o}C, then denatured for 5 min at 65 ^{o}C. The products were separated by electrophoresis on 7% polyacrylamide gel, and visualised under a UV transilluminator. Europeanstone fruit yellows was used as control in the reaction. The RFLP patterns obtained revealed three major phytoplasma groups after comparison with previously published profiles of other phytoplasmas (Lee *et al*., 1998; Marcone*et al*., 2000). These were the SrXIIgroup (Stolbur) in 76.6% samples tested, theSrI group (Aster yellows) in 59.6% samples, and the SrV group (Elm yellows) in 6.6% samples.A high proportion of combination profiles (38.3%) wasobserved (see Figure 3).Two out of three aforementioned groups namely groups SrI and SrV were identified on tomato from Mauritius before (Gungoosingh-Bunwaree*et al*., 2007). These phytoplasma groups have been reported on tomato in several countries abroad and similar techniques were used for their detection (Sertkaya*et al*., 2007; Velios and Lioliopoulou, 2007 and Del Serrone*et al*., 2001). However, this is the first time that Stolbur group SrXII is being reported on tomato in Mauritius and quite surprisingly it was found to be the most prevalent phytoplasma group under local conditions. However, due to the high percentage of mixed infections it was quite difficult to associate visible symptoms on tomato plants with any specific phytoplasma group.

Furthermore, threeamplicons from two stolbur infected samples, were cleaned using *Qiagen* PCRPurification Kit (*Qiagen GmbH*, Hilden, Germany, EU), and then sequenced after identification of polymorphism with *Tru*Irestriction enzyme on R16(I)F/RI primers.Amplicons were samples 2 and 2 bis (different PCR products from same sample) and sample 11 (see Figure 4). Sequences obtained are given in Annex II.

Samples 2 and 2bis showed identical sequences in the two amplicons. Moreover sample 11 showed 99% homology with sample 2 however with 4 mismatches.The obtained 16S rDNA

sequences were subjected to virtual restriction fragment length polymorphism (RFLP) analysis using the *pDRAW32 1.0*, *Revision 1.1.110*, *AcaClone s*oftware (http://www.acaclone.com), as described by Wei *et al*. (2007), in order to determine genetic relatedness . After restriction digestion, a 4.0% agarose gel electrophoresis image was plotted automatically. Figures 5and 6 show pictures of the sequenced amplicons with *Tru*I and *Alu*I respectively.

Results of virtual RFLP analyses with *Alu*I on amplicons obtained from tomato samples after sequencing and alignment indicate that sample 2 is infected by a strain of stolburphytoplasma distinguishable from all others reported worldwide (see figure 6).

Furthermore, maximum parsimony analysis using the close neighbour interchange algorithm was performed with MEGA version 4 (Tamura *et al*., 2007) to construct a phylogenetic tree from the aligned 16S ribosomal sequences and related sequences from different phytoplasma strains. Bootstrap analysis was performed and replicated 1,000 times for estimation of stability and support for the clades. *Acholeplasmalaidlawii* (a cultivable *Mollicute*, phylogenetically related to phytoplasmas) was designated as the out-group to root the tree.

Finally, the phylogenetic tree given inFigure 7 shows that tomato phytoplasma strains 2, 2bis and 11 cluster with otherstolbur isolates. However stolbur strain from sample 2 could be clearly differentiated from that of sample 11 and also from previously detected stolburphytoplasmas from onion and watercress in Mauritius (Gungoosingh-Bunwaree*et al*., 2010).Furthermore,stolbur strain from sample 11 shared a high degree of homogeneity with *Allium cepa* strain OnM2 bearing *GenBank*accession number GU129974. This could be explained by the fact that the aforementioned strain was detected from an onion seed production plot at Richelieu and tomato sample 11 was also collected from that locality.

vii. Determining source and mode of transmission of phytoplasmas at field level

Plastic yellow sticky traps were placed in suspected phytoplasma infected tomato plantations in order to collect insect vectors (Trebicki *et al.,* 2010; Pastore*et al*., 2004). In open fields, yellow traps were fixed in the ground on 50 cm high wooden stakes with the traps oriented towards the wind direction. After one week exposure traps were brought to the laboratory where suspected leaf hoppers were removed and stored in 70% ethanol before their

identities were confirmed. Hence traps were placed in 3 localities (Deux Bras, Richelieu and Trianon) under open field and at RochesBrunes under shadehouse conditions where traps were fixed among and above plants. Howeveroverallnumber of leafhoppers collected was quite low. Only 14 leafhoppers were collected from the 4 localities. Leaf hoppers identified were *Empoasca*, *Balclutha*, *Amrasca* and *Afrolestes* spp.The first three aforementioned leafhoppers are reported to transmit phytoplasma diseasesin tomato elsewhere (Khatri*et al*., 2007, Sertkaya*et al*., 2007, Pantoja*et al*., 2008).The relatively small number of leafhoppers collected could be attributed to the fact that once growers observed abnormal growth in their tomato plants they usually started carrying out regular spraying with insecticides thus resulting in a drastic decline in pest population. Otherwise it could be that the colour or type of trap used was not appropriate. In a study carried out recently (Thein*et al*., 2011), blue sticky traps were found to be as effective as yellow sticky traps for attracting sugar cane white leaf phytoplasmaleaf hopper vectors and light traps were more attractive than sticky traps. Therefore an evaluation of different colours and types of traps prior to the trial could have enabled identification of the most appropriate trap which would attract the highest number of tomato phytoplasmavectors. Furthermore, phytoplasma infection could have been seedborne and then propagated from one plant to another during cultural practices, especially when using unsterilized shears. The last hypothesis could account for high incidences of phytoplasma diseases observed from time under protected conditions. However till date there is only one official report mentioning the possibility of transmission of aster yellows and stolburphytoplasmas from tomato seeds (Calari*et al*., 2011). Therefore it would be interesting to further investigate this hypothesis.

Leaf hoppers were also collected live by the sweeping technique but none of them survived under captivity. Symptomatic weeds collected in the vicinity of phytoplasma positive tomato plants were tested negative to phytoplasmas by nested-PCR. Weeds like amaranthus have been reported to be important hosts of phytoplasmas and viruses in Slovakia (Tothova*et al*., 2004). However since weeds were tested negative under local conditions, symptoms observed on the weeds could be the result of mechanical damage by leaf hoppers or the phytoplasma titre might be too low for detection at the time of testing.

viii. Management of phytoplasma diseases at field level.

During the islandwide survey, as soon as suspected phytoplasma infected plants were spotted, planters were requested to rogue them out and they were told to observe strict sanitation at field level. Growers were also encouraged to use yellow sticky traps (see Plate 5).Moreover growers were advised to prepare their seedlings under insectproof nets and to avoid transplanting young healthy seedlings in the vicinity of older, infected plantations.

Under shadehouse conditions, growers were requested to ensure that their net was not torn so as to keep away all leafhoppers and to use yellow sticky traps. This is because it was noticed during the survey that wherever yellow sticky traps were being properly used phytoplasma incidence was nil (see Plate 6). Growers were also advised to prune out the first branches to show phytoplasma symptoms using shears sterilised in 70% alcohol before and after use, and especially when moving from one plant to another. Since symptoms are usually seen at growing tips or in new plant material, it was suggested to prune stems back up to 2 cm in healthy tissue. This measure could help in limiting phytoplasma spread under shadehouse condition, provided prevailing temperature was unfavourable for rapid multiplication of phytoplasmas in plants and vectors were absent.

Conclusion/recommendations

The islandwide survey carried out over 18 months and across 79 tomato plantations revealed phytoplasma diseases (`maladie pompon' in local jargon) to be widespread in the island. This is because phytoplasma symptoms were observed in 74.6% tomato plantations visited across eight districts of Mauritius. Flacq was the only district where no phytoplasma disease was observed. Moreover even if the overall mean phytoplasma incidence obtained was only 18.1%, the existing risk from these pathogens cannot be underestimated because potential leafhopper vectors were also collected from tomato plantations and phytoplasma diseases can spread fast in situations of poor management.

Phytoplasmas could be detected from tomato leaves and green fruits from symptomatic plants.Three phytoplasma groups namely Stolbur group SrXII, Aster yellows group SrI and Elms yellows group SrV were identified from tomato leaf and fruit samples by nested-PCR followed by RFLP techniques. A high proportion of mixed phytoplasma infections was observed

(38.3%). Phytoplasmas of the Stobur group identified for the first time on tomato in Mauritius, were found to be the most prevalent phytoplasmas on tomato under local conditions. Strain differences were equally detected among the Stolbur isolates infecting tomato locally. Furthermore, mixed phytoplasma and virus infections (PVY and TYLCV) were detected on tomato by the ELISA technique for the first time in Mauritius.

Use of insect-proof nets for seedling preparation, good sanitation measures at field and under shadehouse conditions along with utilisation of yellow sticky traps were recommended as useful management techniques for these emerging plant pathogens.

Future work

1. Evidence for seed transmission of phytoplasmas needs to be confirmed for tomato varieties used under local conditions.
2. The role of leafhoppers as vectors of phytoplasma disease in tomato and the part played by weeds as a reservoir of phytoplasma diseases need to be determined.
3. Local tomato growers need to be sensitized further on the need to follow management techniques mentioned before in order to avoid economic losses due to phytoplasma diseases.
4. Alternate hosts of phytoplasmas identified in this study have to be established.

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I certify to the best of my knowledge (1) the statement herein (excluding scientific hypotheses and scientific opinion) are true and complete, and (2) the text and graphics in this report as well as any accompanying publications or other documents, unless otherwise indicated, are the original work of the signatories or of individuals working under their supervision. I understand that wilfully making a false statement or concealing a material fact in this report or any other communication submitted to MRC is a criminal offense.

Principal Investigator Signature:	**Date:**

MRC Form 1050

ANNEX I

Table 1: Summary of islandwide phytoplasma survey findings

Locality	Area planted (A/m^2)	Tomato variety	Planter`s name	Symptoms observed	Phytoplasma incidence (%)	Severity (%)
Plaine Sophie	0.5A	Swaraksha	Dabee	Plant stunting, bushiness	10	50
Mapou	0.5A	Swaraksha	Mapou MF	Leaf curling, purple colouration	5	40
Réduit	0.5A	MST/32/1	Reduit CRS	Nil	Nil	Nil
Richelieu	0.5A	Swaraksha	Richelieu CRS	Nil	Nil	Nil
Réduit	0.5A	MST/32/1	Réduit CRS	Bushiness, stunting, chlorosis	30	50
Réduit	0.5A	MST/32/1	Réduit CRS	Bushiness, stunting	40	50
Le Chaland	1.5A	Swaraksha	Venkiah	Bushiness, leaf curl	5	40
Le Chaland	1.0A	Swaraksha	Venkiah	Bushiness, stunting	10	20
Union Vale	1.0A	Swaraksha	Venkiah	Leaf curling, purple colouration	5	20
Plaisance Land Settlement	0.75A	Swaraksha	Hurdoyal	Nil	Nil	Nil
Plaisance Land Settlement	1.0A	Swaraksha	Kalka	Bushiness	5	20
Bel Ombre	1.0A	Swaraksha	Jaunkee	Bushiness	5	20
Bel Ombre	2.0A	Swaraksha,Typhoon, Venus rose	Naraina	Stunting, bushiness	10	20
Bel Ombre	1.5A	Swaraksha	Mosafir	Nil	Nil	Nil

Table 1 contd from p16

Mont Blanc	1.0A	Swaraksha	Dowlut	Bushiness	10	20
Martinière	1.0A	Swaraksha	Venkatasami	Bushiness	10	20
Martinière	0.5A	Swaraksha	Unuth	Nil	Nil	Nil
St Avold	1.0A	Swaraksha	Simadree	Bushiness	5	10
Camp Diable	0.5A	Swaraksha	NA	Stunting, bushiness	5	10
St Avold	1.0A	Swaraksha	Ramessar	Nil	Nil	Nil
St Avold	1.0A	Swaraksha	Bholah	Stunting, bushiness	10	20
Camp Diable	0.5A	Pêche rose	Hawoldar	Bushiness	5	10
Grande Chartreuse	0.5A	Pêche Rose	Ramjaun	Stunting, bushiness, purple colouration	25	50
Floréal	0.5A	Swaraksha	Luchoomun	Stunting, purple colouration	80	60
Roches Noires	1.0A	Swaraksha	Neerputh	Nil	Nil	Nil
Roches Noires	1.0A	Pêche Rose	Seernama	Nil	Nil	Nil
Hermitage	1.0A	Swaraksha	Rookmin	Stunting, bushiness	2	20
Hermitage	1.0A	Swaraksha	Samabarti	Stunting	2	20
Schoenfield	2.5A	Swaraksha	Ganawa	Stunting	1	20
Astroea (Eau Bleue)	5A	Swaraksha	Chungoonah	Nil	Nil	Nil
Wireless	2A	Swaraksha	Chungoonah	Nil	Nil	Nil
GrosBillot	1A	Swaraksha	Hurdoyal	Stunting, purple colouration	40	50
Deux Bras	0.5A	Swaraksha	Ackloo	Stunting, purple colouration	40	50
Roches Noires	1.0A	Swaraksha	Neerputh	Nil	Nil	Nil

Table 1 contd from p13

Roches Noires	1.0A	Pêche Rose	Seernama	Nil	Nil	Nil
Deux Bras	0.5A	Swaraksha	Sachin Jankee	Stunting, bushiness, purple colouration	20	30
Deux Bras	1A	Swaraksha	S. Jankee	Stunting, bushiness	20	40
Deux Bras	0.5A	Swaraksha	NA	purple colouration	15	25
Morc St André	0.5A	Swaraksha	Ramchurn	Stunting, bushiness	10	20
Esperance Trébuchet	1A	Swaraksha	NA	Stunting, bushiness	20	25
Esperance Trébuchet	1A	Swaraksha	NA	Stunting	20	25
Poudre d`or village	0.5A	Swaraksha	Madoo	Stunting, bushiness, purple colouration	5	20
Nouvelle Découverte	0.5A	Swaraksha	Ali	Stunting, bushiness	10	20
Nouvelle Découverte	1.0A	Swaraksha	Bungaroo	Stunting, bushiness	10	20
La Laura	0.5A	Swaraksha	Amjad	Stunting, bushiness	5	20
La Laura	0.5A	Swaraksha	Dewkurrun	Stunting, bushiness	5	20
La Laura	0.5A	Swaraksha	Deenowa	Stunting, bushiness	10	20
Camp Diable	0.5A	Swaraksha	Peerbocus	Stunting, bushiness	10	20

Table 1 contd from p16

Tamarin (shadehouse)	2A	Epoch/Menara	Kisna	Leaf distortion, stem elongation	75	75
Tamarin (open field)	3A	Epoch	Kisnah	Nil	Nil	Nil
Trianon	1A	Swaraksha	Somoo	Leaf distortion,	20	25
				stem elongation		
Pailles	0.5A	Swaraksha	Gungaram	Stunting, bushiness	10	20
St André	0.5A	Valentine	Unknown	Nil	Nil	Nil
St André	0.25A	Valentine	Bundoo	Stunting, bushiness	5	20
St André	0.5A	Valentine	Neerooa	Stunting, bushiness	5	20
Bon Air	0.5A	MST/32/1	Chaitee	Stunting, bushiness	5	25
Cotia	0.5A	Valentine	Chumun	Stunting, bushiness	10	25
Fond du Sac Rd	0.5A	MST/32/1	Chumun	Stunting, bushiness	10	20
Médine La Mecque	5A	Epoch	Medine S.E	Leaf distortion, stem elongation	2	25
Tamarin (open field)	3A	Epoch	Medine S.E	Stunting, bushiness	10	25
Tamarin (shadehouse) – 2nd visit	2A	Epoch/Menara	Medine S.E	Leaf distortion, stem elongation	100	95
Terre Rouge	1A	Swaraksha	NA	Stunting, bushiness	10	30
Nouvelle Découverte	0.5A	Swaraksha	Ali	Stunting, bushiness, purple colouration	5	20

Table 1 contd from p15

Trianon	1A	Swaraksha	Somoo	Stunting, bushiness	25	50
Cluny	300m^2 (shadehouse)	Synergie	Lucette	Nil	Nil	Nil
Cluny	300m^2 (shadehouse)	Synergie	Johnson	Nil	Nil	Nil
Union Park	300m^2 (shadehouse)	Synergie	Ramjeet	Nil	Nil	Nil
Deux Bras	300m^2 (shadehouse)	Mathias	Ducasse	Stunting, bushiness	Nil	Nil
Deux Bras	300m^2 (shadehouse)	V169	Ducasse	Nil	Nil	Nil
Bramsthan	600m^2 (shadehouse)	Franceska	Chinatar	Nil	Nil	Nil
Richemare	600m^2 (shadehouse)	Mathias	Bahorun	Nil	Nil	Nil
Poste de Flacq	600m^2 (shadehouse)	V169	Caniah	Nil	Nil	Nil
Roches Brunes	300m^2 (shadehouse)	Synergie	Li	Yellowing, Stunting, bushiness	30	30
Réduit CRS	0.25	MST/32/1	AREU	Stunting, bushiness	1	30
Richelieu	0.2	MST/32/1	AREU	Purple colouration, leaf distortions	60	30
Vale	1500m^2 (5 shadehouses)	Synergie	Chukowree	Stunting, bushiness	1	20

Table 1 contd from p16

Roches Brunes (2nd visit)	300m^2 (shadehouse)	Synergie	Li	Yellowing, Stunting, bushiness, purple colouration	75	30
Richelieu CRS (2nd visit)	0.2	MST/32/1	AREU	Purple colouration, leaf distortions	75	40
No. of Plantations visited: 79 **No. of localities visited**: 30	**Total area covered**: 71.7A	**No. of varieties surveyed**: 12	**No. of planters visited**: 66	**Common symptoms**: Stunting, bushiness, purple colouration	**Mean phytoplasma incidence**: 18.1%	**Mean phytoplasma severity**: 16.6%

Plate 1: Suspected phytoplasma infected tomato plant on right (var. *MST/32/1*). On the left, healthy plant.

Plate 2: Leaf curling and purple colouration on suspected phytoplasma infected plant, var. *Swaraksha*.

Plate 3: Nightshade plant (Brède martin) with purple colouration collected in the vicinity of symptomatic tomato plant

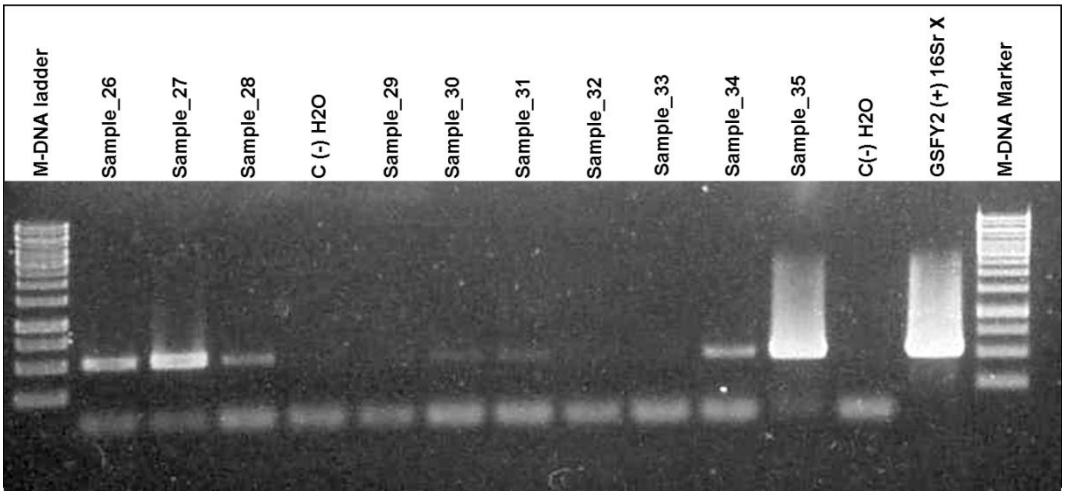

Figure 1: Nested PCR results with primers M1/M2, M: 1Kb DNA ladder, C: control, GSFY2: positive control.

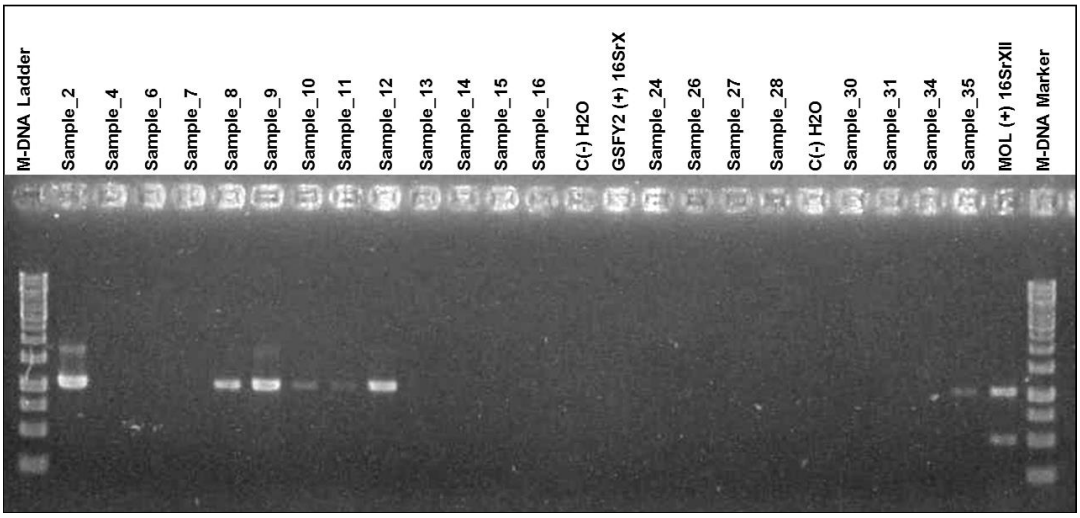

Figure 2: Nested PCR results with primers R16(I) F1/R1, M: 1Kb DNA ladder, C: water control, GSFY2 and MOL: positive controls.

Plate 4: Tomato var. *Swaraksha* plant with mixed phytoplasma and PVY infections

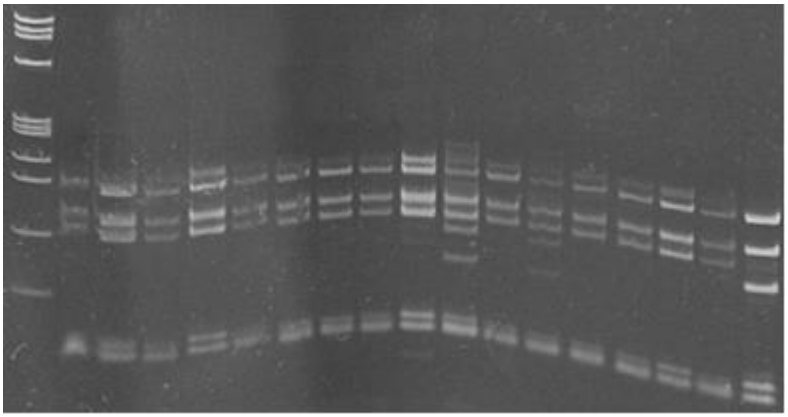

Figure 3: Results of RFLP analyses with *Tru*I on amplicons obtained after amplification with primers M1/M2. Last lane on right is European stone fruit yellows employed as reference. First lane on left is 1Kb DNA marker.

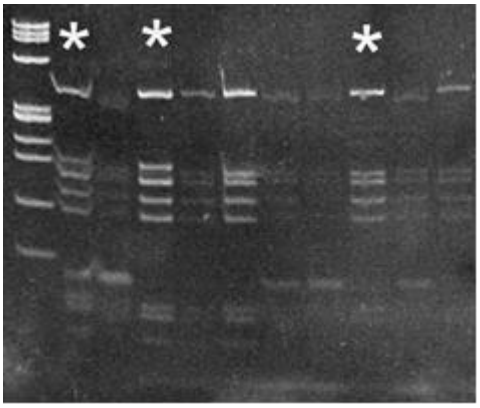

Figure 4: Results of RFLP analyses with *Tru*I on amplicons obtained from tomato samples after amplification with primers R16(I) F1/R1: stolbur phytoplasmas are in all samples. Lanes marked with stars are the polymorphic profiles of samples selected for sequencing. RFLP profiles of first and second star from left are from the same sample 2 and 2bis.

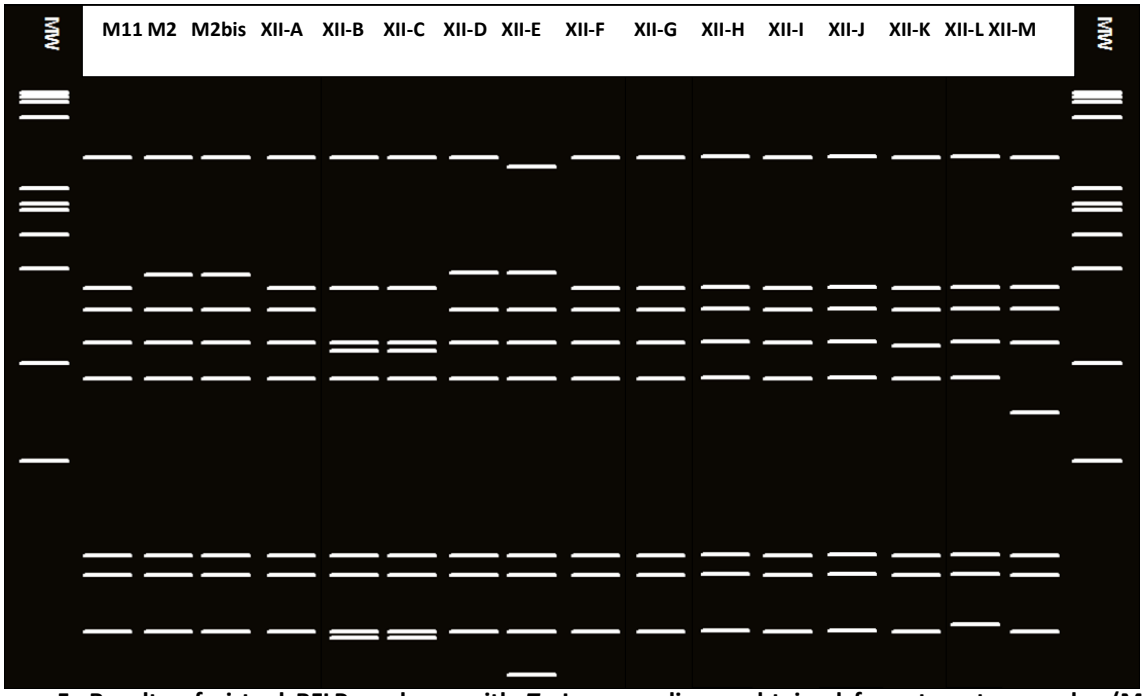

Figure 5: Results of virtual RFLP analyses with *Tru*I on amplicons obtained from tomato samples (M11, M2 and M2bis) after sequencing and alignment. RFLP profiles of first three samples from left are those from stolbur phytoplasma in tomatoes. AF248959=XII-A, L76865=XII-B; AJ243045=XII-C; AB010425=XII-D; DQ086423=XII-E; EU836651=XII-F; EU836646=XII-G; EU010007=XII-H; EU010008=XII-I; EU014777=XII-J; DQ222972=XII-K; EU131021=XII-L; DQ160245= XII-M.

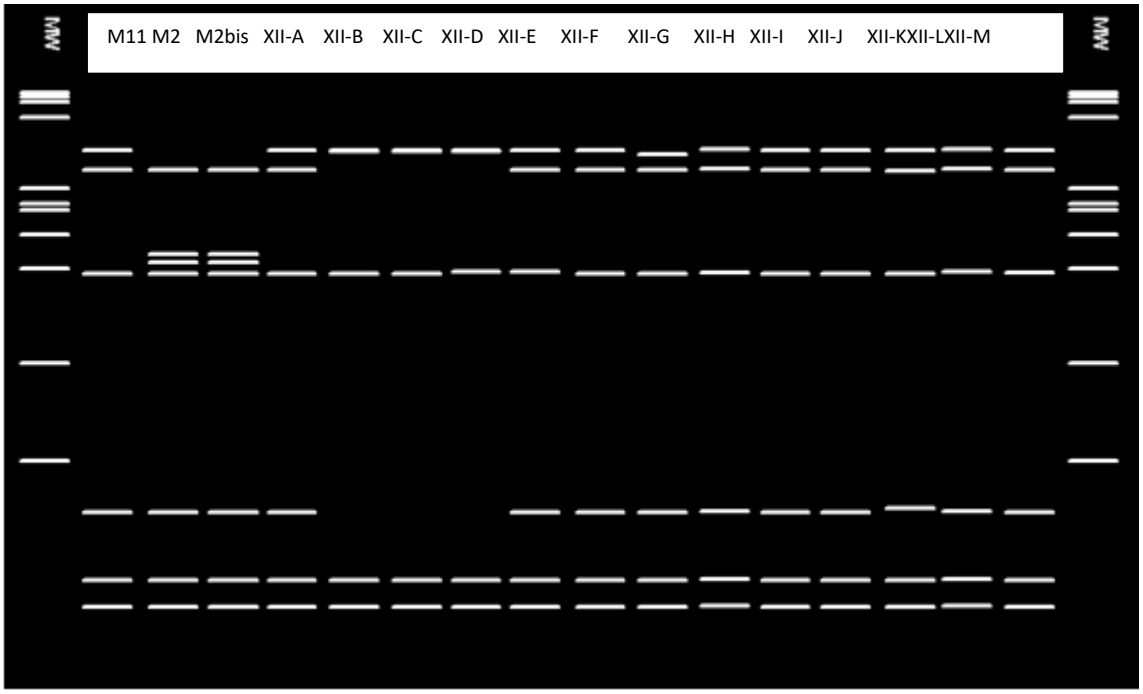

Figure 6: Results of virtual RFLP analyses with AluI on amplicons obtained from tomato samples after sequencing and alignment. RFLP profiles of first three samples from left (M11, M2 and M2 bis) are those from stolbur phytoplasma in tomatoes from Mauritius and show that sample 2 is infected by phytoplasmas that are distinguishable from all other reported worldwide. AF248959=XII-A, L76865=XII-B; AJ243045=XII-C; AB010425=XII-D; DQ086423=XII-E; EU836651=XII-F; EU836646=XII-G; EU010007=XII-H; EU010008=XII-I; EU014777=XII-J; DQ222972=XII-K; EU131021=XII-L; DQ160245= XII-M.

Figure 7: Phylogenetic tree with the 3 stolbur phytoplasmas sequenced from tomato (indicated with red arrows) and from onion and watercress (indicated with blue arrows) from Mauritius. GenBank accession numbers are given, where available.

Plate 5: Placement of yellow sticky traps in phytoplasma infected tomato plantation of var. *Swaraksha*

Plate 6: Tomato shadehouse free from any phytoplasma symptoms. Yellow sticky traps used for insect control.

Sequences of 3 Stolbur amplicons

Tomato sample 2 (1 095 bp)

TAAAAGACCTAGCAATAGGTATGCTTAGGGAAGAGCTTGCGTCACATTAGTTAGTTGGTGGGGTAATGGCCTACCAAGACGATGATGTGTAGCCGG
GCTGAGAGGTCGAACGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAAGGAATTTTCGGCAATGGAGGAAACTCTG
ACCGAGCAATGCCGCGTGAACGATGAAGTATTTCGGTACGTAAAGCTCTTTTATTAGGGAAGAAAAGATGGTGGAAAAACCATTATGACGGTACCT
AATGAATAAGCCCCGGCTAACTATGTGCCAGCAGCCGCGGTAATACATAGGGGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGGGTGCGTAGG
CGGTTAAATAAGTTTATGGTCTAAGTGCAACGCTCAACGTTGTGATGCTATAAAAACTGTTTAGCTAGAGTTGGATAGAGGCAAGTGGAATTCCGTG
TGTAGTGGTAAAATGCGTAAATATACGGAGGAACACCAGAAGCGAAGGCGGCTTGCTGGGTCTTAACTGACGCTGAGGCACGAAAGCGTGGGGA
GCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGAGTACTAAACGTTGGATAAAACCAGTGTTGAAGTTAACACATTAAGTACTCCG
CCTGAGTAGTACGTACGCAAGTATGAAACTTAAAGGAATTGACGGGACTCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGGTACCCGAAAA
ACCTCACCAGGTCTTGACATGCTTTTGCAAAGCTGTAGAAATACAGTGGAGGTTATCAGAAGCACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCG
TGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTGTTAATTGCCATCATTACGTTGGGGACTTTAGCAAGACTGCCAATGATAAATTGG
AGGAAGGTGGGGACGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACAAACGTGATACAATGGCTGTTACAAAGGGTAGCTAAAGCGTAAGC
TTCTGGCGAATCTCAAAAAAGCAGTCTCAGTTCGGATTG

Tomato sample 2bis (1 095 bp)

TAAAAGACCTAGCAATAGGTATGCTTAGGGAAGAGCTTGCGTCACATTAGTTAGTTGGTGGGGTAATGGCCTACCAAGACGATGATGTGTAGCCGG
GCTGAGAGGTCGAACGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAAGGAATTTTCGGCAATGGAGGAAACTCTG
ACCGAGCAATGCCGCGTGAACGATGAAGTATTTCGGTACGTAAAGCTCTTTTATTAGGGAAGAAAAGATGGTGGAAAAACCATTATGACGGTACCT
AATGAATAAGCCCCGGCTAACTATGTGCCAGCAGCCGCGGTAATACATAGGGGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGGGTGCGTAGG
CGGTTAAATAAGTTTATGGTCTAAGTGCAACGCTCAACGTTGTGATGCTATAAAAACTGTTTAGCTAGAGTTGGATAGAGGCAAGTGGAATTCCGTG
TGTAGTGGTAAAATGCGTAAATATACGGAGGAACACCAGAAGCGAAGGCGGCTTGCTGGGTCTTAACTGACGCTGAGGCACGAAAGCGTGGGGA
GCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGAGTACTAAACGTTGGATAAAACCAGTGTTGAAGTTAACACATTAAGTACTCCG
CCTGAGTAGTACGTACGCAAGTATGAAACTTAAAGGAATTGACGGGACTCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGGTACCCGAAAA
ACCTCACCAGGTCTTGACATGCTTTTGCAAAGCTGTAGAAATACAGTGGAGGTTATCAGAAGCACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCG
TGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTGTTAATTGCCATCATTACGTTGGGGACTTTAGCAAGACTGCCAATGATAAATTGG
AGGAAGGTGGGGACGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACAAACGTGATACAATGGCTGTTACAAAGGGTAGCTAAAGCGTAAGC
TTCTGGCGAATCTCAAAAAAGCAGTCTCAGTTCGGATTG

Tomato sample 11 (1 095 bp)

TAAAAGACCTAGCAATAGGTATGCTTAGGGAAGAGCTTGCGTCACATTAGTTAGTTGGTGGGGTAATGGCCTACCAAGACGATGATGTGTAGCCGG
GCTGAGAGGTCGAACGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAAGGAATTTTCGGCAATGGAGGAAACTCTG
ACCGAGCAATGCCGCGTGAACGATGAAGTATTTCGGTACGTAAAGTTCTTTTATTAGGGAAGAAAAGATGGTGGGAAAACCATTATGACGGTACCT
AATGAATAAGCCCCGGCTAACTATGTGCCAGCAGCCGCGGTAATACATAGGGGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGGGTGCGTAGG
CGGTTAAATAGGTTTATGGTCTAAGTGCAACGCTCAACGTTGTGATGCTATAAAAACTGTTTAGCTAGAGTTGGATAGAGGCAAGTGGAATTCCGT
GTGTAGTGGTAAAATGCGTAAATATACGGAGGAACACCAGAAGCGAAGGCGGCTTGCTGGGTCTTAACTGACGCTGAGGCACGAAAGCGTGGGG
AGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGAGTACTAAACGTTGGATAAAACCAGTGTTGAAGTTAACACATTAAGTACTCC
GCCTGAGTAGTACGTACGCAAGTATGAAACTTAAAGGAATTGACGGGACTCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGGTACCCGAAA
AACCTCACCAGGTCTTGACATGCTTTTGCAAAGCTGTAGAAATACAGTGGAGGTTATCAGAAGCACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTC
GTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTGTTAATTGCCATCATTAAGTTGGGGACTTTAGCAAGACTGCCAATGATAAATTG
GAGGAAGGTGGGGACGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACAAACGTGATACAATGGCTGTTACAAAGGGTAGCTAAAGCGTAA
GCTTCTGGCGAATCTCAAAAAAGCAGTCTCAGTTCGGATTG